Role of Fibrinopeptide B in Early Atherosclerotic Lesion Formation*

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The development of atherosclerotic lesions involves many cell types, including macrophages. Fibrinopeptide B (FPB) has been shown to be a potent chemotactic agent for macrophages, which are abundant as intimal foam cells in atherosclerotic lesions, especially in cholesterol-fed rabbits. We hypothesize that intimal low-density lipoproteins also cause fibrinogen in the intima to release FPB and that FPB attracts macrophages in response to the high lipid levels associated with lesion development. To test our hypothesis, we used an atherosclerotic model. Silk sutures containing either FPB, fibrinopeptide A (FPA), lipopolysaccharide (LPS), or saline control were prepared. One suture of each type was placed in the adventitia of the femoral artery of a rabbit. Animals were killed at 1 or 2 weeks. Only vessels exposed to either FPB or LPS showed significant intimal thickening in the region adjacent to the suture site. Semi-thin electron microscopic sections indicated that the intimal wall was highly cellular and that many cells contained lipid vacuoles after 2 weeks. These sections also showed that the endothelium remained intact and that no injury to the media of the artery had occurred. Electron microscopy of the tissue samples showed the proliferation of smooth muscle cells and deposition of extracellular matrix in the 2-week animals, whereas foam cells were present in the 1-week animals. We conclude that FPB does indeed attract macrophages to the intima and that these macrophages may become foam cells. The model we have developed can be used to study possible mechanisms for the entry of macrophages into the intima during early lesion development and to further understand the complex interactions of FPB, fibrinogen, and lipids in atherosclerotic lesion development.

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The development of atherosclerotic lesions is a complex process and probably involves interactions between circulating blood elements, including serum lipids and white blood cells (e.g., monocytes and lymphocytes), and the arterial wall, including endothelial cells and smooth muscle cells of the media. The nature of this interaction and the role of blood-borne cells in this process, especially macrophages, are not completely understood.

Fibrinogen is a serum protein with a major role in blood clotting. It may also be the source of a chemotactic factor for the monocytes that accumulate as intimal foam cells during atherogenesis. Three considerations point to such a mechanism. First, diet-induced hyperlipemia is associated with an increased influx of apo-B-containing lipoproteins into the intima [*1,2*], and hypertension increases the influx of low-density lipoprotein and fibrinogen into the arterial wall [*3*]. Second, the gelatinous intimal swellings that are thought to precede lesion development are rich in fibrinogen and fibrin [*4*], and a close association has been shown between fibrinogen and apo-B-containing lipoproteins in the arterial wall [*5,6*]. Third, upon binding to loosely packed phospholipid surfaces, fibrinogen polymerizes to fibrin and two peptides are released: fibrinopeptide B (FPB) and fibrinopeptide A (FPA). FPB has been found to be a potent chemotactic agent for macrophages [*7,8*], the intimal foam cells within atherosclerotic lesions. Thus, a causative association between hyperlipemia and the release of FPB could provide a basis for the generation of foam cell lesions in cholesterol-fed animals.

The complex process involving the interactions of fibrinogen, FPB, and lipoproteins is not fully understood. One possible mechanism is that when fibrinogen binds to lipoproteins with the requisite phospholipid surface, the lipids become oxidized so that they cannot exit the vessel wall. Since there is also a release of FPB, we hypothesize that FPB attracts macrophages to deal with the offending agent (the altered lipids).

Previous studies by Prescott *et al* [*9*] demonstrated focal intimal thickening after placement of sutures containing lipopolysaccharide (LPS), a component of bacterial cell walls and a strong polyclonal activator of B lymphocytes, along an arterial wall. The purpose of this project was to develop and test a similar model to study the role of FPB in early atherosclerotic lesion formation. FPB may be the source of a chemoattractant for macrophages, which accumulate as intimal foam cells during atherogenesis. Having established a suitable model, our next step will be to investigate whether the mechanism we have proposed can be shown to be a factor in atherogenesis.

MATERIAL AND METHODS

Sixteen New Zealand White rabbits (3.0 to 3.5 kg) were used. 4.0 silk sutures were prepared by soaking them

in solutions of each of the following compounds: FPA, 0.1 mg/mL in saline; FPB, 0.1 mg/mL in saline; LPS, 2 μg/mL in saline; control suture in saline (no peptide).

The sutures were soaked in the solution overnight at room temperature for 12 hours in a small chamber vacuum and then dessicated in a framehood. The compound-embedded sutures were routinely sterilized (by gassing) for surgical implantation.

Rabbits were preanesthetized with ketamine (30 to 40 mg/kg) and Xylazine (5 mg/kg). They were anesthetized by inhalation of halothane (1% to 2.5%). The left and right femoral arteries were exposed. A needle was used to implant the suture material through a 5-mm segment of the adventitial tissue of each femoral artery. The suture was then tied in a loop to hold it in place and the ends were trimmed. The incisions were then closed. The animals recovered from surgery shortly afterwards. Following surgery, the rabbits were fed a high-cholesterol diet of rabbit chow with 4% corn oil and 1% cholesterol. Serum cholesterol levels were obtained at the time of death for half the animals. Two femoral arteries were harvested at 1 week for each type of suture material (except for the control group in which only one artery was successfully sacrificed at the time period due to surgical difficulties), and the remaining arteries were harvested at 2 weeks for each type of suture material. Arteries and their respective suture types were assigned randomly.

After sacrifice, the vessels were fixed by perfusion (at physiologic mean pressure) for 30 minutes using glutaraldehyde (3%). The arteries were then excised and prepared for histologic examination. The suture and material were carefully removed from the tissue, and the location on the vessel wall was marked with India ink. Three different histologic stains (hematoxylin and eosin, Weigert von Giesson, and Gomori-trichrome aldehyde fucsin) were used to prepare the histologic sections.

Semi-thin electron microscopic sections were also prepared to further analyze the cells present in the intima. The slides were also used to determine whether the endothelium remained intact and if the media had been injured.

All sections were examined under a light microscopic, and images were projected onto a Numonics 2200 digitizing tablet. An 80286-based microcomputer utilizing digitizing software was used to measure the intimal thickening. The thickness of the intima was measured at its thickest point. This was found to be adjacent to where the suture material had been inserted. No thickness was found in any other region of the vessel in all the arteries. Values for each group were combined and analyzed by treatment groups. Analysis of variance was performed on the means ($p = 0.0264$) followed by Student's unmatched *t*-tests.

RESULTS

Due to the high cholesterol diet, serum cholesterol levels were elevated in all animals at the time they were killed. Rabbits killed 1 week after surgery had 20-fold increases in cholesterol levels (1,192 ± 400 mg/dL), whereas those killed 2 weeks after surgery had nearly 30-fold increases in cholesterol levels (1,474 ± 270 mg/dL).

TABLE I
Mean Intimal Thickness for Each Suture Type

Variable	Control (μm)	FPA (μm)	FPB (μm)	LPS (μm)
Number (n)	7	8	8	8
Mean	5.08	4.75	10.16	16.99
Maximum	6.30	8.38	21.56	51.08
Minimum	3.10	2.59	3.78	5.23
SD	1.37	2.07	5.76	15.37

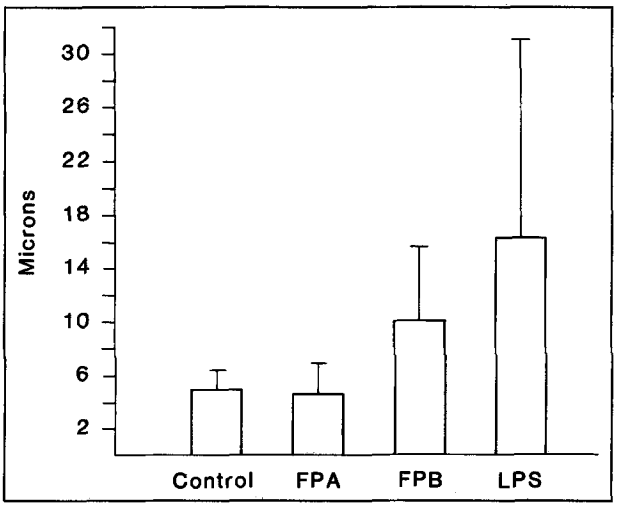

Figure 1. Bar graph comparing the means of intimal thickness for each treatment group with SD.

The control and FPA sutures failed to cause any substantial focal intimal thickness in their adjacent femoral arteries. Arteries containing control sutures or FPA sutures in the adventitia had a mean intimal thickness of 5.08 ± 1.37 μm and 4.75 ± 2.07 μm, respectively. The mean intimal thickness of the FPB sutures was calculated at 10.16 ± 5.76 μm, almost twice the size of the FPA or control sutures. The LPS sutures (used as a positive control) caused a mean intimal thickness of 16.99 ± 15.37 μm (Table I). The mean intimal thickness along with standard deviation bars for each suture type are graphically displayed in Figure 1. The difference in intimal thickness between the FPB:control ($p = 0.0409$) and FPB:FPA ($p = 0.0255$) sutures was found to be significantly different.

Ultrastructure evaluations indicated that the endothelium remained directly on the internal elastic lamina in those arteries exposed to FPA or control sutures (Figure 2). The ultrastructure of the FPB site at 2 weeks demonstrated the presence of smooth muscle cells, foam cells, and extracellular matrix (Figure 3). Arteries exposed to FPB for 2 weeks contained more smooth muscle cells and fewer foam cells than those examined at 1 week.

COMMENTS

The results indicate a significant increase in the intimal thickening in arteries exposed to either LPS- or FPB-impregnated sutures as compared with control sutures. The mean intimal thickness of those arteries containing FPB or LPS sutures was almost two to three times the

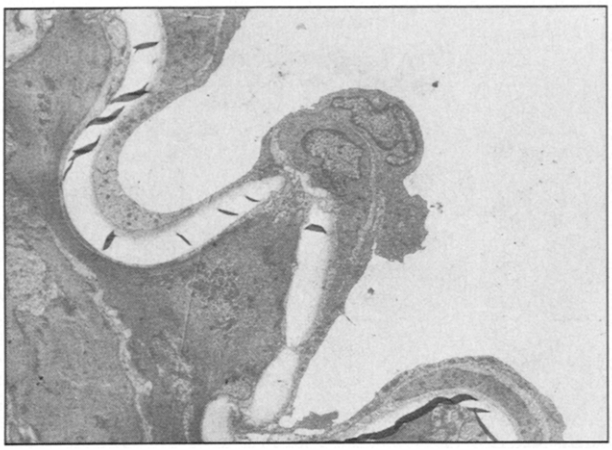

Figure 2. High-power electron micrograph showing an endothelial cell lying above the internal elastic lamina. This artery was exposed to a FPA suture and thus fails to show any intimal thickening (original magnification × 6,100, reduced by 50%).

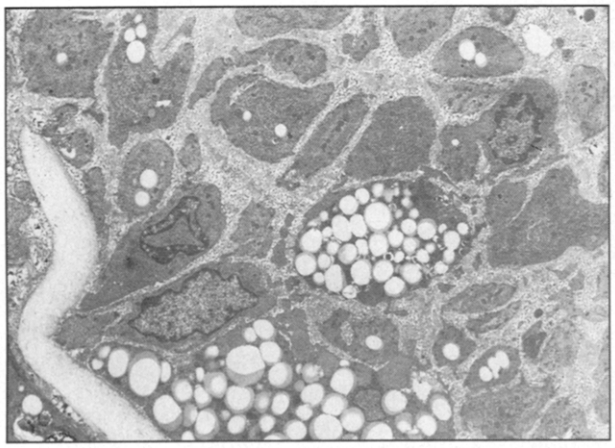

Figure 3. Electron micrograph of a thickened intima with infiltration of smooth muscle cells, foam cells, and extracellular matrix in response to a FPB suture at 2 weeks (original magnification × 6,100, reduced by 50%).

control value. We expected the control sutures to show no intimal thickening since they contained no peptide. FPA also did not cause any intimal thickness that could be observed histologically. The failure of FPA to elicit a response enabled us to conclude that it does not play a direct role in causing intimal thickness.

LPS was shown by Prescott *et al* [9] to be effective at causing intimal thickening. LPS in our model yielded similar results. There was a great amount of variability in the LPS suture reaction due to a 1-week animal failing to respond as the others did. This artery caused the great variability shown by the standard deviation brackets. LPS was used in our experiment to demonstrate that the reactivity of the artery wall was not compromised by our procedure. Had LPS failed to cause intimal thickening, we would have had to question the sensitivity of the procedure to elicit a response. Nevertheless, the fact that we were able to duplicate Prescott's work provided support that our modified model was successful.

The most important compound in this study was FPB. There was obvious focal intimal thickening in all arteries exposed to FPB sutures. The thickness of the lesions was very similar to that with LPS. This thickness was once again present only in the region closest to the suture. FPB was definitely shown to cause a significant difference in intimal thickness when compared with the control or FPA sutures.

Ultrastructure studies revealed that the intimal thickening due to FPB and LPS sutures contained small clusters of intimal foam cells along the internal elastic lamina, smooth muscle cells, and also lipid vacuoles. We suggest that these lipid vacuoles were probably macrophages that internalized the surrounding lipid. Intimal foam cells were only present in the vessel that contained FPB or LPS sutures. The FPA and control suture arteries did not exhibit increased intimal thickening and therefore no intimal foam cells were present. Unlike other surgical models, histologic examination showed that the endothelium remained intact and that no obvious injury had occurred to the artery's medial layer. The presence of foam cells in the vessels that underwent intimal thickening provides support for the hypothesis that FPB attracts macrophages possibly to remove the lipids that enter the vessel.

FPB has been shown in these experiments to be associated with increased intimal thickening. The presence of FPB in a region adjacent to the vessel wall attracts macrophages to that site. The mechanism by which the intima thickens is not understood. A possible explanation is that macrophages are unable to withstand the high levels of lipids that are present in the artery and therefore begin releasing their proteolytic enzymes. These enzymes lead to local tissue destruction and, therefore, lesion development. The next stage of this project is to understand the actual role of macrophages once they have been attracted to the vessel wall and to further understand the role of hyperlipidemia in the mechanism. The fact that we have confirmed FPB's role as a macrophage attractant and have developed a focal model for further studies is a step toward understanding this complex stage of atherosclerotic lesion development.

In conclusion, in these experiments, we showed that FPB led to the recruitment of macrophages in the form of foam cells to the intima of arteries focally. This finding supports one of our initial claims concerning the development of lesions. With the model we have developed in this experiment, we can now explore other aspects of lesion development (e.g., the role of high lipid levels, the role of fibrinogen, and the mechanism by which macrophages cause lesion development). By further modifing our model and experimental technique, we are confident we can enhance our data. We may now be able to explore and understand the complex interactions involved with atherosclerotic lesion development more precisely and thoroughly.

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